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Increased exposure to yolk testosterone has feminizing effects in chickens, *Gallus gallus domesticus*

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Competing for food by altricial and semiprecocial bird nestlings is a behaviour well known for its sensitivity to maternal androgens during prenatal development. Whether a similar effect is present in precocial species that do not beg is less well known. We therefore increased yolk testosterone levels within the physiological range at the onset of incubation to study its effects on food competition behaviour in the domestic chicken, *Gallus gallus domesticus*. We found an increase in competitiveness in testosterone-treated male domestic chicks, raising their level to that of the females. This is in line with the decrease in circulating plasma levels of males in the direction of the levels in females, and the overall decrease in androgen receptor densities after prenatal treatment as found previously. Hormones are known to have long-lasting organizing effects on behaviour and to affect sexual differentiation in vertebrates. Although research into hormone-mediated maternal effects has been productive, only a few studies describe (the ambiguous) effects into adulthood. Therefore we followed our animals into adulthood and recorded androgen-dependent social behaviour and secondary sexual characteristics, body mass and circulating plasma testosterone levels and checked whether these variables were treatment dependent. Treatment had a near significant effect on comb colour (both brightness and chroma). Again treatment caused a shift towards a more female-like phenotype. This suggests that, in contrast to earlier suggestions, maternal androgens may interact with (but not disrupt) sexual differentiation of brain and behaviour and the development of secondary sexual characteristics.

In many vertebrate species embryos are exposed to steroids of maternal origin (for birds, fish, reptiles and mammals see e.g. Schwabl 1993; McCormick 1999; Bowden et al. 2000; Drea 2011, respectively). Such exposure can organize phenotypic differences within and between the sexes and affects behaviour, physiology and the organization of the brain (Hews & Moore 1995; Gil 2003; Strasser & Schwabl 2004; Groothuis et al. 2005; Daisley et al. 2005; Cohen-Bendahan et al. 2005; Rogers 2006; Eising et al. 2006; Dloniak et al. 2006; Weinstock 2008; Pfannkuche et al. 2009). Exposing offspring to hormones during early development is generally interpreted as a way for mothers to adjust their offspring's phenotype to the prevailing environmental conditions to maximize fitness (Groothuis et al. 2005).

Much research on these hormone-mediated maternal effects makes use of bird species, because bird embryos develop outside the mothers' body in a sealed environment that allows for easy measuring and manipulation of prenatal exposure to steroid hormones. Effects of varying levels of maternal testosterone (T) in egg yolk have received most attention. This focus on T is caused by the finding that egg yolks contain maternally derived androgens (Schwabl 1993) that vary systematically within and between clutches (Groothuis et al. 2005; Gil 2008; von Engelhardt & Groothuis 2011).

Prenatal exposure to T is important since increased yolk T levels can affect important traits in altricial and semiprecocial chicks such as competition, for example begging for parental food provisioning and aggressive behaviours, growth, immunity and survival (Schwabl 1996; Strasser et al. 1998; Eising et al. 2003; Groothuis et al. 2005; von Engelhardt et al. 2006; Rubolini et al. 2006b; Muller et al. 2006, 2009a, b; Gil 2008; von Engelhardt & Groothuis 2011). Several of these effects of yolk T are sex specific; for example in zebra finches, *Taeniopygia guttata*, yolk T affected female but not male begging and growth (von Engelhardt et al. 2006) and in the domestic canary, *Serinus canaria domestica*, it increased post-hatching growth in females, but decreased it in males (Muller et al. 2008, 2009b).

Begging behaviour in altricial and semiprecocial species in particular has received much attention. Such chicks are dependent on their parents for food and compete with siblings for parental

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provisioning by begging. Increased yolk T levels stimulate begging behaviour in several altricial and semiprecocial species (Schwabl 1996; Eising & Groothuis 2003; von Engelhardt et al. 2004; Rubolini et al. 2006b). Although yolk androgens can affect behaviour or morphology in precocial chicks too (Daisley et al. 2005; Okuliarova et al. 2007), the effect on sibling competition has been mentioned (without data being presented) in only one study (see Groothuis et al. 2005). This lack of studies is probably related to the erroneous assumption that precocial chicks do not rely on parental provisioning and the fact that fowl species are studied in laboratory conditions without the parents being present. However, fowl mothers, including the domestic chicken, *Gallus gallus domesticus*, perform tid-bitting displays (Domm 1927) by which they indicate to offspring the location of highly prized food items. This is usually followed by chicks trying to outcompete each other for this item. Therefore the first aim of this study was to test the effect of elevated yolk T on sibling competition in the precocial domestic chick. Based on Groothuis et al. (2005) we predicted that increased exposure to T would increase competitiveness, especially in male chicks.

Prenatal exposure to androgens can also have long-term organizing effects on behaviour and phenotypic traits (Nelson 1995). This is intriguing from both a proximate and an ultimate perspective: First, our knowledge of organizing effects of prenatal exposure to androgens on brain and behaviour in avian species comes mainly from studies on sexual differentiation (Cohen-Bendahan et al. 2005). Recent evidence from egg injection studies using dosages within the physiological range indicates that maternal androgens may affect the phenotype via different pathways from sexual differentiation of the gonads, the reproductive tract, and brain and behaviour (see Carere & Balthazart 2007 and Groothuis & Schwabl 2008 for a discussion on this topic). Second, such prenatal programming suggests that mothers may adjust the offspring's phenotype not only to early life conditions but also to conditions further in the future. Alternatively, maternal modification of the offspring's adult phenotype may be the outcome of a natural selection process on 'adjusted' chick phenotypes.

Studies on any long-term effects of prenatal exposure to steroids on behaviour and phenotypic traits, however, are scarce and present ambiguous results. In almost adult house sparrows, *Passer domesticus*, the size of the black bib, an important sexually selected trait, was increased by prenatal exposure to T (Strasser & Schwabl 2004). Likewise, in 10-month-old black-headed gulls, *Larus ridibundus*, the development of the black mask was accelerated (Eising et al. 2006). However, the effect on the sparrows could not be replicated (Partecke & Schwabl 2008). Moreover, there were no long-term effects on such traits in adult European starlings, *Sturnus vulgaris* (Muller & Eens 2009), a negative effect on spur length in adult ring-necked pheasants, *Phasianus colchicus* (Rubolini et al. 2006a) and delayed song development in adult canaries (Muller et al. 2008). In almost adult canaries, house sparrows and black-headed gulls aggression, competitiveness and dominance were increased (Eising & Groothuis 2003; Strasser & Schwabl 2004; Schwabl 1993; Partecke & Schwabl 2008), but the effect in the canary could not be replicated in adult birds (Muller et al. 2008). We therefore not only tested the effect of increased yolk T level on early sibling competition but also followed these birds into early adulthood. To this end we injected eggs before incubation either with T dissolved in sesame oil or with sesame oil only, taking care that the manipulation was within the physiological range of this species.

Recently it was shown that the same experimental manipulation resulted in a decrease in plasma T levels and androgen receptor densities in the hypothalamic area in 2-week-old chicks (Pfannkuche et al. 2011). The latter finding is especially interesting because, although hormone-mediated effects on behaviour have been extensively researched, much about the underlying mechanisms is as yet unknown. One possibility for how the organizing effects of hormones may come about is via an alteration of the hypothalamus–pituitary–gonadal axis, which would affect endogenous hormone production later in life. Although in quail, *Coturnix japonica,* chicks the effect on plasma T levels was not statistically significant (Daisley et al. 2005), prenatal exposure increased plasma T levels significantly in 3-week-old nestlings of the spotted starling, *Sturnus unicolor* (Muller et al. 2007). A long-term study in the house sparrow, however, did not show an effect of prenatal exposure to androgens on plasma T levels in adulthood (Partecke & Schwabl 2008). Given these results the decrease in T production and sensitivity in the domestic chick is intriguing. We therefore investigated whether the decrease in plasma T levels found in 2-week-old chickens was still present after the onset of reproduction.

Since circulating plasma levels in adult chickens are positively associated with winning fights and social position (Ligon et al. 1990; Johnsen & Zuk 1995), we predicted that elevation of yolk T would result, at least in males, in a lower social position later in life. We also predicted that this would coincide with less pronounced secondary sexual characteristics such as smaller and less red combs and wattles since these are under the influence of circulating T (Ligon et al. 1990; Johnsen & Zuk 1995; Zuk et al. 1995; Verhulst et al. 1999).

METHODS

Treatment and Housing

Freshly laid eggs of laying hens were purchased from a commercial breeder (Verbeek Lunteren, The Netherlands) and incubated at the Zoological Laboratory in Haren (University of Groningen, The Netherlands). We used the LSL strain, a commercially available white leghorn type, which is selected on productivity traits. There is no reason to assume that prenatal exposure to hormones would exert other general effects in this strain more than in other strains. It is relatively easy to keep and there are no large behavioural differences apparent compared to other commercial strains or junglefowl, *Gallus gallus*. The LSL allows for easy neonatal phenotypic sexing of the animals on the basis of feather growth.

Eggs received an intrayolk injection of 75 ng crystalline T dissolved in 100 μl sesame oil or 100 μl sesame oil (C) only. This 75 ng is approximately two times the standard deviation of T measured in a subset of eggs of the same batch of eggs (Groothuis et al. 2005; Pfannkuche et al. 2011). Injecting was facilitated by drilling a small hole in the egg shell which, after injection, was sealed with candle wax. Eggs were incubated at 37.5 °C and 60% humidity, and turned automatically three times a day. At day 18 eggs were reallocated to hatching trays within the same incubator. After hatching in individual compartments, sexing and individual marking, chicks were kept in 12 groups of eight (two chicks of each sex * treatment combination). After 6 weeks a subset of animals of both treatments and sexes was killed for brain analyses (Pfannkuche et al. 2011) and the rest were kept in single-sex and mixed-treatment groups in two large outdoor roofed aviaries (approximately 10 × 20 m and 3.5 m high). In addition to a metal wing clip received early in life, males and females were colour marked (black, blue or green) on the feathers (head, neck, back or a combination of two regions) with a permanent marker at the onset of the observations to enable individual recognition. To decrease the chance of observer bias we took the following measures: (1) colours or coloured regions were not specific for sex or treatment, and within but not between groups colour markings were unique to an individual; (2) there was no information about individual treatment in the housing or

experimental rooms, or on any protocol form other than the one used when collecting the chicks from the incubator.

Throughout the experiment, food (standard laying hen pellet) and water were available ad libitum and three times a week mixed grains were provided as well.

Food Competition in Young Chicks

Five days after hatching, chicks were weighed and assigned to groups of three, matched for sex and weight: two unfamiliar focal chicks (one C and one T chick) and one unfamiliar stimulus chick (randomly assigned from either treatment). In total, 22 male and 22 female groups were formed.

Each pair of focal chicks was placed in the test arena (diameter = 150 cm) behind a transparent barrier. The stimulus chick was placed in the test arena on the other side of the barrier and given a mealworm, *Tenebrio molitor*, while both focal chicks were watching. As soon as the stimulus chick picked up the mealworm, the barrier was removed and the focal chicks were free to 'steal' the mealworm from the stimulus chicks. The frequency of pecks of both focal chicks towards the mealworm in the bill of the stimulus chick was recorded. All groups were tested in three consecutive trials, which were performed without breaks between the trials. The total number of pecks towards the mealworm for each individual was summed for the three trials. Trials ended after the mealworm was swallowed, which occurred on average 11.17 ± 1.06 s after the barrier was removed.

Social Position in Adult Animals

When the birds were between 22 and 26 weeks old we observed all outcomes from overt aggressive interactions (pecking at the head, kicking and chasing) during six 1.5 h sessions per aviary. Losing an interaction was determined by the following behaviours after being 'attacked': (1) moving away quickly from the attacker without retaliating, the head usually held low or (2) assuming an immobile submissive crouching posture. The two groups were composed of seven C and seven T males and six C and nine T females, respectively. In total 247 interactions were scored in the male group and 499 in the female group. From the 91 possible unique male–male dyadic interactions 16 were not observed. The median number of the total number of interactions between two males of these 91 combinations was 2 (1st–3rd quartile 1–4; maximum 16). The median number of interactions of each male was 32 (1st–3rd quartile 28.75–41.25; range 15–71 interactions). In the female group only six of 105 possible unique dyadic interactions were not observed and here the median number of interactions between two females was 4 (1st–3rd quartile 2–7; maximum 26). The median number of interactions of each female was 61 (1st–3rd quartile 55–71; range 38–105 interactions). Because dominance relationships are usually stable, at least over short time periods, the David's score was calculated from all win–lose interactions in order to construct a dominance hierarchy (David 1987; Gammell et al. 2003). This score takes into account both the proportion of interactions won and the number of individuals defeated for an individual relative to the other individuals in the population. The scores were then ordered and the most successful bird within each group was assigned rank 1, the second most successful bird assigned rank 2 and so on.

Biometric Measurements in Adults

All birds were caught, weighed and bled in week 22. In week 26 a photograph was taken of the comb and wattle from a standardized distance and against a millimetre graph paper background in order to determine the size of the comb and wattles. The photographs were uploaded onto a computer where the comb and wattle parts were isolated using Adobe Photoshop 8.0. Then, the number of pixels of comb and wattles were determined using Sigma scan Pro and these values were translated into mm^2 using the pixel count of an area of known size of the graph paper.

We also determined the redness of the comb by measuring the spectral reflectance using a USB-2000 spectrophotometer with illumination from a DH-2000 deuterium halogen light source (both Avantes, Eerbeek, the Netherlands), following the protocol described by Korsten et al. (2007) and Vedder et al. (2008). Three readings were taken of the side of the comb equally spaced over the full length of the comb. The spectrophotometer measured spectral reflectance in the range 200–850 nm in discrete steps of 0.31 nm. Brightness, hue and chroma values were computed using the yellow–red spectrum (400–700 nm). Similar to Casagrande et al. (2011) brightness was calculated as the average reflectance ($R_{average}$) over the 400–700 nm range. Chroma was calculated as $(R_{max}-R_{min})/R_{average}$, where R_{max} was calculated as the average reflectance of the 10 highest points, and R_{min} as the average reflectance of the 10 points with the lowest reflectance. Hue was determined as the spectral location of the midpoint between the average wavelengths of R_{max} and R_{min}. The averages of the three scans were checked for repeatability, the interclass correlation coefficients (r_{27}) of brightness, chroma and hue were 0.98 ($P < 0.001$), 0.91 ($P < 0.001$) and 0.44 ($P = 0.034$), respectively. Because of its low repeatability, hue was ignored in further analyses.

Plasma T Levels in Adults

We took a blood sample within 20 min after catching the first bird in the group. Blood was taken from the wing vein and stored on ice after which the blood samples were centrifuged for 10 min at 9000 rpm. The plasma was collected and stored at −80 °C until hormone extractions and measurements. In total, we obtained sufficient volume in 26 of 29 plasma samples to measure plasma T levels. All plasma samples were measured in one assay (intra-assay variation $2.62 \pm 0.45\%$). Plasma samples and eight pooled samples were weighed and 50 μl radioactively labelled T (Perkin Elmer Life and Analytical Science BV) was added to enable us to control for losses during the extraction process (recovery). After an incubation time of 1 h, 2.5 ml diethyl ether/petroleum benzine (70:30) was added and samples were vortexed and centrifuged. Samples were snap frozen in a mixture of ethanol and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was again dissolved in 1 ml 70% methanol and samples were stored overnight at −20 °C. In the morning, samples were centrifuged, the methanol phase was decanted and the samples dried again under streaming nitrogen. Pellets from samples were resuspended in 95, 100 or 150 μl PBS buffer, depending on the amount of plasma that was available. We used 30 μl of this mixture for measuring recoveries (average recovery rate for T: $78.83 \pm 0.58\%$). Testosterone levels in plasma samples and pooled samples were measured with one radioimmunoassay (RIA) using a commercial kit (Active Testosterone Coated-Tube RIA DSL-4000 kit, Diagnostic Systems Laboratories) with a sensitivity of 0.08 ng/ml T and cross-reactivities of 5.8% with DHT and 2.3% with A4.

Statistical Analysis

The food competition data were not normally distributed (Kolmogorov–Smirnov test: $P < 0.001$) and different transformations did not yield normality. Therefore we used a non-parametric test, comparing treatment groups separately within

males and females. We used the Wilcoxon signed-ranks test because of the paired test design in which groups always consisted of one T and one C chick, matched for sex, age, weight and stimulus bird. Sex differences within treatment groups were tested with a Mann–Whitney *U* test. Stimulus animals were matched for weight and sex, but randomly chosen from each treatment. To test for a possible effect of the treatment of the stimulus animal on the length of the trials and on the number of pecks by each focal animal at the mealworm, Mann–Whitney *U* tests were conducted.

Except for rank order and number of interactions won, all variables measured in the adult stage were normally distributed. Therefore we used ANOVAs with sex and the interaction between sex and treatment as predictor variables for the latter variables. If the interaction term was not significant it was dropped from the model. We used the Mann–Whitney function to test for treatment effects within the sexes separately for effects on social rank order. We used both Spearman rank and Pearson correlations to explore relationships between variables. If there were sex differences in a particular variable, we calculated these correlations for the sexes separately. In both the male and female groups one sample contained T levels that were more than five times the standard deviation above the average of the rest of the same-sex samples. These outliers were therefore not considered during statistical analyses. There was no relationship between sampling order and plasma T levels in either males or females (linear regression: males: $F_{1,10} = 0.676$, $P = 0.432$, $R^2 = 0.070$; females: $F_{1,10} = 0.077$, $P = 0.787$, $R^2 = 0.007$) and sampling order was therefore ignored in further analyses. The general linear model procedure was also used to investigate the relationship between body mass and social position. All tests were two tailed and all calculations were performed using SPSS 16 (SPSS Inc., Chicago, IL, U.S.A.).

Ethical Note

All experiments described in this paper were carried out under DEC licence 4765 of the University of Groningen, the Netherlands. All handling and treatment of animals was carried out by experienced scientists and animal caretakers, with a licence to perform animal experiments. Birds were colour marked (blue, black or green, no red or reddish colours were used) with a nontoxic marker. This did not cause any specific pecking behaviour of other birds. Additionally, they received a ca 2 cm long numbered metal wing clip through the patagium, which caused mild discomfort for about half a minute. Prenatal exposure to T (in the natural range) did not cause any side-effects such as retarded growth or severe aggressive behaviour. Fluctuating T levels were measured in adults by taking up to 250 µl of blood from the wing vein. In none of the tests or housing conditions did severe aggression (prolonged chasing and pecking of 'victims' that obviously showed signs of submission) or injurious pecking occur. The welfare of all birds was assessed on a daily basis throughout the experiments. At all times a recovery space was available (which we did not need to use) for animals suffering from social interactions or otherwise showing illness, impaired wellbeing, injuries, etc. The 2-week-old chicks were killed for brain analyses by direct decapitation; the older birds were first killed with an increasing concentration of CO_2.

RESULTS

Food Competition Test in Young Chicks

The in ovo T treatment was effective, but only in males (Fig. 1): males from T-injected eggs showed higher frequencies of pecking behaviour than C males did (Wilcoxon signed-ranks test: $T = 4.0$, $N = 22$, $P = 0.027$). Within females there was no effect (Wilcoxon signed-ranks test: $T = 36.0$, $N = 22$, $P = 0.811$). C males pecked less than C females (Mann–Whitney *U* test: $U = 156.0$, $N_1 = N_2 = 22$, $P = 0.028$), and T treatment reduced the sex difference that was present in C animals to a nonsignificant level (Mann–Whitney *U* test: $U = 223.5$, $N_1 = N_2 = 22$, $P = 0.638$).

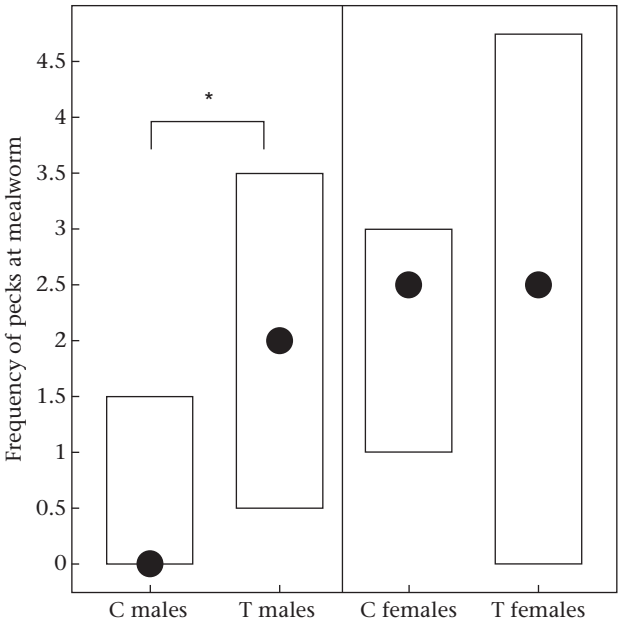

Figure 1. Frequency of the total number of pecks at the mealworm in three mealworm competition tests, separated for sex and treatment. Dots represent the median and bars represent the 25th to 75th percentiles of the total number of pecks where there was a winner (ties not included). $^*P < 0.05$.

These results were independent of the treatment of the stimulus animal, since there were no effects of treatment on either the average length of trials (Mann–Whitney *U* test: $U = 179.0$, $N_1 = N_2 = 22$, $P = 0.166$) or the number of pecks at the mealworm or stimulus animal (Mann–Whitney *U* test: C animals: $U = 208.0$, $N_1 = N_2 = 22$, $P = 0.447$; T animals: $U = 235.5$, $N_1 = N_2 = 22$, $P = 0.959$).

Social Position and Body Mass in Early Adulthood

The number of interactions won (Table 1; Mann–Whitney *U* test: males: $U = 21$, $N_1 = N_2 = 7$, $P = 0.653$; females: $U = 24.0$, $N_1 = 6$, $N_2 = 9$, $P = 0.723$) and the social position (Table 1; Mann–Whitney *U* test: males: $U = 18$, $N_1 = N_2 = 7$, $P = 0.406$; females:

Table 1
Characteristics of adult layer chickens prenatally treated with testosterone.

	C males (7)	T males (7)	C females (6)	T females (9)
Interactions won	17; 11–33	14; 2–28	27.5; 11–48.3	37; 16.5–49.5
Social position (rank)	7; 2.75–11.25	8; 5–12.5	5; 2–12	8; 1–14
Body mass (g)	1725±43	1706±59	1432±54	1410±28
Comb & wattle size (mm^2)	4988±330	4478±558	552±151	314±26
Plasma T levels (ng/ml)	0.69±0.13 (5)	0.71±0.07 (6)	0.45±0.01 (6)	0.40±0.02 (7)

The table shows rank order variables (rows 1 and 2 median; first to third quartile) and biometric variables (rows 3–5, mean ± SEM) separated for sex and treatment (C: vehicle injection in ovo; T: testosterone injection in ovo). Rank 1 signifies the most dominant animal, 2 the second most dominant, etc. Sample sizes for each group and for plasma T levels are given in parentheses.

$U = 23$, $N_1 = 6$, $N_2 = 9$, $P = 0.637$) were independent of treatment within both the male and the female groups.

There was no significant interaction effect between sex and treatment on body mass; this term was therefore dropped from the model (ANOVA interaction: $F_{1,28} = 0.002$, $P = 0.967$). Males were heavier than females and there was no effect of prenatal treatment on body mass (ANOVA: sex: $F_{1,28} = 41.36$, $P < 0.001$; treatment: $F_{1,28} = 0.20$, $P = 0.656$, $R^2 = 0.633$). Pooling the data of the treatments showed that males with a higher social position were heavier than subordinate males and this was nearly significant (Fig. 2; general linear model: $F = 4.389$, $N = 14$, $P = 0.058$, $R^2 = 0.268$), and that there was a significant quadratic relationship between rank and body mass in females (Fig. 2; $F = 6.784$, $N = 15$, $P = 0.011$, $R^2 = 0.531$). The residual body masses of these relationships were also not dependent on treatment (ANOVA: $F_{1,28} = 0.091$, $P = 0.766$).

Secondary Sexual Characteristics

There were no significant interaction effects between sex and treatment on the size of the comb and wattles, the comb's brightness or chroma. Only the main effects therefore remained in the model: males had a larger comb and wattle than females but treatment had no effect (see Table 1; ANOVA: sex: $F_{1,28} = 178.5$, $P < 0.001$; treatment: $F_{1,28} = 1.35$, $P = 0.256$, $R^2 = 0.88$). Female combs were brighter than male combs and treatment had a near significant positive effect on comb brightness (Fig. 3, ANOVA: sex: $F_{1,28} = 106.9$, $P < 0.001$; treatment: $F_{1,28} = 3.86$, $P < 0.06$, $R^2 = 0.817$). Female combs had a lower chroma and treatment had a near significant negative effect on chroma (ANOVA: sex: $F_{1,28} = 26.1$, $P < 0.001$; treatment: $F_{1,28} = 3.71$, $P < 0.06$, $R^2 = 0.553$).

Male ornament size was positively correlated with body mass and increasing social position (body mass: Pearson $r_{12} = +0.71$, $P = 0.004$; social position: Spearman $r_S = +0.79$, $P = 0.001$), negatively with brightness, but not with chroma (brightness: Pearson $r_{12} = -0.58$, $P = 0.031$; chroma: Pearson $r_{12} = +0.39$, $P = 0.170$). Female ornament size was not correlated with body mass or social position (body mass: Pearson $r_{13} = +0.29$, $N = 15$, $P = 0.765$; social position: $r_S = +0.04$, $P = 0.879$) but was correlated with both brightness and chroma (brightness: Pearson $r_{13} = -0.806$, $P < 0.001$; chroma: Pearson $r_{13} = +0.59$, $P = 0.017$).

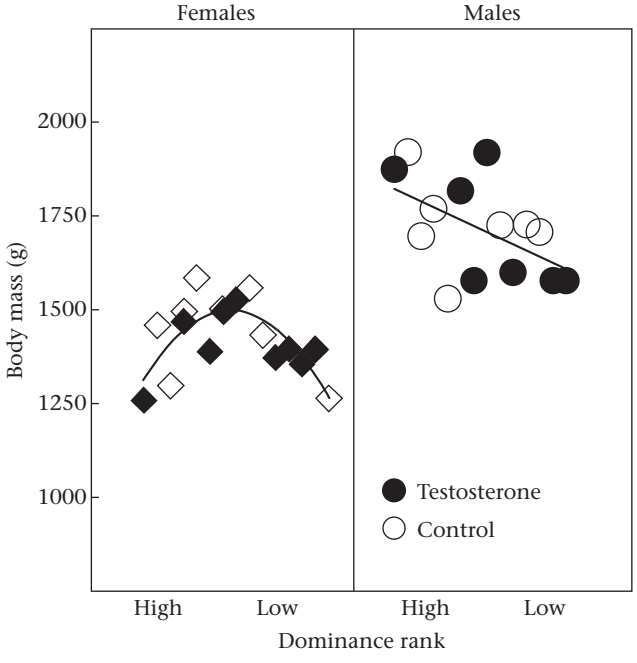

Figure 2. The relationship between body mass and dominance rank in female (line $y = 1254 + 64.5x - 4.25x^2$) and male (line $y = 1839 - 16.4x$) laying chickens.

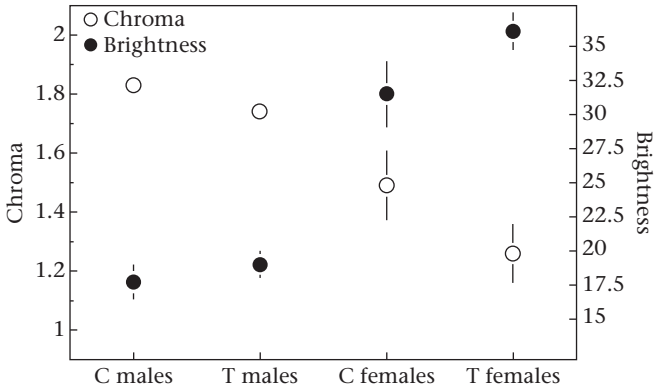

Figure 3. Comb chroma and brightness in adult male and female chickens prenatally treated with T and in controls (C). Error bars indicate SEs.

Plasma T Levels

There was no significant interaction effect of sex and treatment on plasma T levels (ANOVA: $F_{1,23} = 0.131$, $P = 0.722$) and this term was therefore dropped from the model. Females had lower plasma T levels than males (Table 1: males: 0.70 ± 0.09 ng/ml; females: 0.42 ± 0.03 ng/ml; ANOVA: $F_{1,23} = 9.718$, $P = 0.005$), but there was no effect of prenatal treatment (ANOVA: $F_{1,23} = 0.027$, $P = 0.871$).

Within males or within females there were no significant correlations between plasma T levels and social position, comb colour, comb and wattle size or body mass, respectively (Spearman: $-0.52 < r_S$ values $< +0.49$, all P values > 0.1).

DISCUSSION

The aim of this study was to evaluate the effects of elevated maternal T on food competition in a precocial species, which lacks the begging displays of altricial and semiprecocial birds, and to study long-term organizing effects of this treatment in reproductively active chickens. Pfannkuche et al. (2011) showed that circulating plasma T levels in the blood in young male domestic chicks shifted towards more female-like levels when yolk T levels were increased within the physiological range just prior to the onset of incubation. Moreover, this treatment decreased androgen receptor densities in both males and females and seemed to do more so in males than females, towards a female level, although this could not be tested because of technical differences. In this study we showed that this shift towards female-like levels is also apparent in competitive behaviour early in life. Moreover, our results on comb colour characteristics indicate that this effect may not be transient but may last into adulthood: T-treated males tended to have more female-like combs. It may be that these feminizing effects of T on physiology, behaviour and secondary sexual characteristics are caused by the conversion of yolk T to oestrogen early in life (Nelson 1995), which may affect the process of sexual differentiation of brain and behaviour. This is in contrast to the suggestion that only pharmacological doses of T and not levels within the physiological range in the yolks of fresh and unincubated eggs may affect the process of sexual differentiation of brain and behaviour (Carere & Balthazart 2007; Groothuis & Schwabl 2008) and this needs further study.

Food Competition

This is the first documented report showing that maternal T can stimulate competitive behaviour in a precocial species. The test showed that elevated maternal T (1) enhanced pecking behaviour towards a mealworm in the possession of a competitor in young male domestic chicks, (2) raising their level of competitiveness to the level of the female chicks. Several studies have shown that yolk T can stimulate begging behaviour in altricial and semiprecocial bird species, affecting both the postural and vocal displays (canaries: Schwabl 1996; gulls: Boncoraglio et al. 2006; Eising & Groothuis 2003; zebra finches: von Engelhardt et al. 2004). The increase in begging behaviour is interpreted as improving the position of the chick in the competitive hierarchy of the brood. While precocial chicks do not perform conspicuous begging displays, yolk T facilitated their ability to compete for a food item, a normal part of their repertoire when raised by their mother, who often presents food items to her chicks.

A sex-specific effect of yolk T on begging has been tested in only two studies. In zebra finches, only females showed more begging behaviour after treatment with prenatal T. Males showed overall higher frequencies of begging behaviour (von Engelhardt et al. 2004), so that the lack of effect on male chicks may be caused by a ceiling effect. The opposite seemed to be the case for young chickens in the present study: among control birds, females pecked more than males. Confirming Groothuis et al. (2005), the in ovo T treatment in the present study decreased this sex difference by elevating competitive behaviour in males, but not in females. This might also be caused by a ceiling effect in females. The mechanism underlying the sex-specific effect, for example a higher sensitivity to T caused by the higher density of androgen receptor densities in males, and the possible ceiling effect in females, requires further study. Other sex-specific effects on behaviour have been found, for example in the pied flycatcher, *Ficedula hypoleuca*, in which increased yolk T affected exploration and antipredator behaviour in males but not females, and also recruitment rates (Ruuskanen & Laaksonen 2010; Ruuskanen et al. 2012b). These sex-specific effects are intriguing also from a functional perspective, as they may provide mothers with a way of adjusting the secondary sex ratio. Muller et al. (2002) showed that laying hen mothers provide male and female eggs differentially with T, depending on their rank position in the social hierarchy. That finding also suggests that mothers may adjust the quality of sons and daughters to the rearing environment using differentially allocated maternal hormones, maximizing their fitness return via sons and daughters (Trivers & Willard 1973). In the collared flycatcher, *Ficedula albicollis*, however, increased yolk T levels decreased the recruitment rate of sons (but not daughters). This 'fitness' decrease in sons was not compensated for by an increased attractiveness or breeding success (Ruuskanen et al. 2012a, b), Daughters also did not benefit from enhanced yolk T levels indicating that the earlier suggestions need adjustment for predicting long-term effects of prenatal exposure to androgens on fitness returns.

Long-term Effects on Social Position and Body Mass

In contrast to results found in the house sparrow (Strasser & Schwabl 2004; Partecke & Schwabl 2008), the black-headed gull (Eising et al. 2006) and possibly the spotless starling (Veiga et al. 2004), but similar to results in the canary (Muller et al. 2008), pied flycatcher (Ruuskanen & Laaksonen 2010) and ring-necked pheasant (Bonisoli-Alquati et al. 2011a), there was no long-term effect of prenatal exposure to increased yolk T level on winning social interactions or on dominance status in our study. This might have been because our study animals were housed outdoors in large aviaries during the autumn when daylength was shortening. In addition, average plasma T levels in junglefowl are still increasing at this age (Johnsen & Zuk 1998) and the levels we measured were relatively low. Nevertheless, overt aggressive interactions were relatively frequent, especially in females. In contrast to the studies mentioned above, we scored only those interactions with overt aggression and not those that involved only minor threat displays and this may also explain the discrepancy with other studies. The lack of an effect of our treatment on social position and winning fights is supported by the lack of effect on body mass and T levels, which often correlate positively with these social parameters (see below).

In birds there are indications that prenatal T treatment plays a role in postnatal growth and development (Schwabl 1996; Eising et al. 2001, 2003; Rubolini et al. 2006a, b; Ruuskanen et al. 2012a) but the literature is not entirely consistent (see von Engelhardt & Groothuis 2011). Although data on adult body mass are generally lacking, body mass at fledging was not different even in the studies showing an effect of body mass in early development (Schwabl 1996; Eising et al 2001). In our experiment there was also no effect of treatment on adult body mass. Perhaps this is because prenatal T treatment affects growth only via begging and competitiveness in birds during parental provisioning (Boncoraglio et al. 2006). After this period there is ample time for control birds to catch up with their prenatally T-exposed conspecifics, especially in the affluent rearing conditions we provided.

Body mass is often correlated with social position (Gottier 1968; Cloutier & Newberry 2000). Usually dominant fowl are heavier than subordinates (Cloutier & Newberry 2000; Muller et al. 2002). In our male group this was indeed the case. This confirms that our estimates of social position scored via the method of David (1987) are probably biologically relevant. In females there was a concave relationship between body mass and social position. This finding is different from the relations that, for example, Muller et al. (2002) found. However, their social groups contained a rooster, which can modulate social interactions among the hens (McBride 1964; Craig & Bhagwat 1974; Oden et al. 1999, 2000). There are several possible explanations why in our single-sex groups high-ranking females weighed less than females in the middle of the hierarchy: dominant females (1) can afford to have smaller reserves and thus a lower body mass because they can always dominate resources (Ekman & Lilliendahl 1993; Ekman 2004), (2) expend much more energy in maintaining their social position and (3) spend more energy on reproduction (Muller et al. 2002). On the other side of the relationship, lower ranking animals (1) may be less strong or (2) were prevented from foraging by dominant animals. In any case, our study does not support any large long-term effects of exposure to prenatally increased T levels on social position and body mass later in life.

Long-term Effects on Secondary Sexual Characteristics

So far, increased prenatal yolk T levels have been found to increase the black bib size in the house sparrow (Strasser & Schwabl 2004), induce earlier moult into the nuptial plumage in black-headed gulls (Eising et al. 2006) and increase wattle hue in the non-UV part of the visible spectrum in the ring-necked pheasant (Bonisoli-Alquati et al. 2011b). However, the first effect could not be replicated by Partecke & Schwabl (2008), whereas in the ring-necked pheasant (Rubolini et al. 2006a; but see Bonisoli-Alquati et al. 2011b), the Chinese quail, *Coturnix chinensis* (Uller et al. 2005), the European starling (Muller & Eens 2009) and the collared flycatcher (Ruuskanen et al. 2012a) there were either no effects on secondary sexual characteristics or only a suppression of spur length in the pheasant (Rubolini et al. 2006a). In our study, as

expected, comb and wattle size, a sexually selected trait (Zuk et al. 1990, 1995; Cornwallis & O'Conner 2009), of males was larger than that of females. Likewise, brightness was lower and chroma higher in males than females, which indicates the reliability of our measurements. T treatment did not affect comb and wattle size but affected almost significantly brightness and chroma in a more female direction. This has not been shown before and the increased brightness and decreased chroma would make males more female like in appearance, perhaps reducing male attractiveness for the other sex. In ring-necked pheasants, however, prenatal T exposure reduced the number of copulations in females but increased it in males even though it did not affect other (sexually selected) traits (Bonisoli-Alquati et al. 2011a) apart from wattle hue (Bonisoli-Alquati et al. 2011b). As in our study, chroma also decreased nearly significantly. It is unclear whether comb characteristics are either feminized or masculinized by treatment since the females of this species have no wattles.

Our finding is consistent with the finding that early in life our treatment decreased the androgen receptor densities in the brain, shifting the male phenotype more towards the female phenotype (Pfannkuche et al. 2011); this shift towards the female phenotype was also present in plasma T levels and competitive behaviour, both early in life.

Male ornament size increased with body mass and rank. This finding is in line with the robust relationship between comb/wattle size and dominance (Zuk et al. 1995; Verhulst et al. 1999). In females these relationships were not apparent, but comb colour correlated with size. In females there are also indications that comb and wattle characteristics are under sexual selection (Cornwallis & O'Conner 2009).

Long-lasting Effects on Circulating Plasma T Levels

Previously Pfannkuche et al. (2011) showed that there are two possible pathways through which the effect of prenatal exposure to T may act on the phenotype of 2-week-old chickens: a decrease in circulating plasma T levels and simultaneously a down regulation of androgen receptor densities in the brain. However, the first effect seems only temporary since we could not detect a treatment-dependent long-lasting effect on circulating plasma T levels. As mentioned above, this might be caused by a suppressing effect of short daylength and the absence of females on T production; alternatively, levels may fluctuate too much on a daily basis under the influence of social interactions for any effects of treatment to be detected by a single sample. Unfortunately we were unable to analyse androgen receptor densities in our adult chickens, but studies on the dynamics of androgen receptor, and other hormone receptor, densities in the chicken brain during growth would be very helpful in understanding the mechanisms of hormone-mediated maternal effects.

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